miR-290 acts as a physiological effector of senescence in mouse embryo fibroblasts

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Pitto L, Rizzo M, Simili M, Colligiani D, Evangelista M, Mercatanti A, Mariani L, Cremisi F, Rainaldi G. miR-290 acts as a physiological effector of senescence in mouse embryo fibroblasts. Physiol Genomics 39: 210–218, 2009. First published September 1, 2009; doi:10.1152/physiolgenomics.00085.2009.—The culture-induced senescence of mouse embryo fibroblasts (MEF) correlates with reduction of cell proliferation. In this work we found that the accumulation of cells with 4C DNA content and the transcriptional change of several microRNAs (miRNAs or miRs) are relevant events in culture senescence. By comparing the miRNA expression profiles of physiologically senescent MEF and that of senescent MEF induced by the downregulation of leukemia-related factor, we identified miR-290 as a common upregulated miRNA. When miR-290 was transfected in presenescent MEF, SA-β-gal cells and p16, two markers of culture senescence, increased compared with control, indicating that miR-290 is causally involved in senescence. Interestingly, nocodazole (NCZ), which induces G2/M block, increased the percentage of senescent cells as well as the expression of miR-290 and of the tumor suppressor p16, thus mimicking culture senescence. As miR-290 was overexpressed in NCZ-treated cells and it was able to induce senescence in proliferating MEF, we investigated whether miR-290 and NCZ could share common mechanisms of culture senescence. Whereas the induction of SA-β-gal+ by miR-290 was not strengthened by coupling its transfection with NCZ treatment, the transfection of the antagonist 290 (d-290) plus NCZ treatment, while blocking cells at G2/M, suppressed SA-β-gal+ and p16 induction. On the basis of these findings we conclude that miR-290 might act as a physiological effector of NCZ induced as well as culture senescence via p16 regulation expanding the role of this miRNA from embryonic stem to differentiated cells.

microRNAs; nocodazole; cell cycle

CELLULAR SENECESSCE REPRESENTS a stress response whereby cells undergo irreversible cell cycle arrest (18). In mouse embryo fibroblasts (MEF) senescence can be induced by cellular stresses such as the persistent mitogenic stimulation during propagation in culture (10), the overexpression (32) or downregulation (20) of single oncogenes, or DNA-damaging drugs (37). Most senescence-dependent cellular dysfunctions are the result of a complex interaction between gene and environment over a cell life span. Genes identified as associated with cellular senescence can be grouped into families regulating redox/homeostasis (7, 34), DNA repair (19), apoptosis (10), and growth factor signaling (6). Deciphering how these genes interact to control senescence might help to elucidate the signaling networks at the basis of senescence. MicroRNAs (miRNAs or miRs), a class of endogenous 22–25 nt single-stranded RNA molecules that bind to the 3’-untranslated region of multiple messenger RNAs and inhibit translation (4, 5), represent a network of factors that, together with transcription factors, exerts a spatial and temporal control of gene expression with the final effect depending on the sum of the affected genes and on the genetic background of the cell. With the development of microarrays that examine the expression profiles of miRNAs, the overall contribution of miRNAs to senescence can be approached.

The culture senescence of MEF is a well-described model (10). We used this model to investigate if senescence is accompanied by changes in the expression of specific miRNAs and whether these miRNAs have a causative role in inducing senescence.

METHODS

Reagents. We list here the reagents and their sources: mature miR-290, miR-100, miR-125b, miR-NC (negative control, double-stranded oligonucleotide designed to serve as negative control in miRNA mimic experiments), the antagonist 290 (d-290), d-100, d-125b, d-NC (inhibitor negative control, single-stranded oligonucleotide designed to serve as negative control in miRNA inhibition experiments) (GenePharma, Shanghai, China), Gene Silencer (Gene Therapy Systems, San Diego, CA), oligoFITC (IFC, Pisa, Italy), TRizol reagent, DNasel amplification grade, SuperScript II reverse transcriptase, Dulbecco’s modified Eagle medium-high glucose (DMEM-HG), Optimum, fetal bovine serum (FBS, Invitrogen), miScript System, RNeasy mini kit (QIAGEN, Milan, Italy), LightCycler 480 Probes Master, Universal ProbeLibrary LNA Probes; LightCycler 480 SYBR Green I Master (Roche Diagnostic, Mannheim, Germany), X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside), propidium iodide (PI), crystal violet, nocodazole (NCZ; Sigma-Aldrich, St. Louis, MO), Exiqon miCURY LNA Array version 8.1, labeling method of miRNAs (Exiqon), phosphorylated histone H3 (PH3) antibody (Millipore), anti-leukemia-related factor (anti-LRF; BDIMC, Boston, MA), anti-E2F1 (Santa Cruz Biotechnology), enhanced chemiluminescence (ECL, Amersham).

Cells and culture conditions. Wild-type MEF were isolated from 13.5 day mouse embryos. Briefly, embryos were mechanically fragmented and then incubated with trypsin (0.25% in PBS pH 7.5) and continuously stirred at 37°C for 15–20 min. After 10 min centrifugation at 290 g, pellets were resuspended in DMEM-HG without FBS and centrifuged for 10 min at 290 g. After three washings, the cell suspensions were distributed in culture dishes containing DMEM-HG +10% FBS. Cells were trypsinized at confluence (passage 1). The propagation protocol 6T3 (6 × 10⁶ cells/100 mm diameter dish transferred every 3 days) was followed. MEF were grown at 37°C in a humidified atmosphere containing 6% CO₂. At each passage cellular and molecular endpoints were detected.
Senescence-associated β-galactosidase activity. Samples of 2 × 10^5 cells were seeded in 30 mm diameter dish, and 24 h later dishes were washed once with PBS and fixed for 3–5 min at room temperature with PBS containing 2% formaldehyde/0.2% glutaraldehyde. Cells were then washed three times with PBS and incubated at 37°C with fresh senescence-associated (SA)-β-galactosidase (gal)-"staining solution containing 1 mg/ml X-Gal (stock = 20 mg/ml in DMSO), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2 in PBS pH 6.0. After 24 h cells were washed in PBS and stained with 20 µg/ml Hoechst 33342 for 10 min. Dishes were scored with a Leica ILDM inverted microscope to count SA-β-gal" cells and Hoechst-positive nuclei. The ratios SA-β-gal" cells/Hoechst-positive nuclei of 25 consecutive fields (~500 cells) were used to determine the frequency of the event.

DNA staining by PI. Samples of 6 × 10^5 cells were stained with PI according to standardized procedure. DNA content profiles were detected by flow cytometry and fold changes calculated as specified in figure legends.

miRNA microarray. To choose miRNAs involved in senescence, the miRNA expression profiles of presenescent (PRE SEN) and senescent (SEN) MEF were performed as followings. Total RNA (2 µg) was labeled and manually hybridized to Exiqon miRCURY LNA Array 8.0, following the manufacturer’s protocol. Differential labeling of total RNA samples with dyes spectrally equivalent to Cy3TM and Cy5TM fluorophores allowed comparison of miRNA expression patterns of PRE SEN MEF (passage 2) (internal reference) and SEN MEF (passage 6). The labeling method allows selective labeling of miRNA in the total RNA sample. The hybridized microarrays were scanned with a GenePix 4000B instrument, and data were acquired and analyzed with GenePix Pro software. Data were normalized with print-tip Loess method by the CARMAweb application developed at the Institute for Genomics and Bioinformatics of Graz University of Technology (30).

Expression analysis of miRNAs. Total RNA was extracted from 1 × 10^6 cells with TRIzol following the manufacturer’s recommendation. Mature miR-290, miR-291–3p, miR-292–3p, miR-295, miR-100, and miR-125b were quantified using the miScript System according to the manufacturer’s instructions. Oligonucleotides 5'-AACCCGTAGATCGACCGTTCTGG-3', 5'-TCCCTGAGACCTGAACTT-3', 5'-CCTGGAGAAACCTGCCAAGTATGATG-3', 5'-AGGGTCTC-3', 5'-CGACGGGCATAGCTTC-3' were used as forward primers, respectively, for miR-100, miR-125b, miR-290, miR-292–3p, miR-295 and U6 in the real time amplification mixtures. All reactions were performed in triplicate and data were analyzed as described (see “Real-time PCR analysis”) using U6 as internal control. The relative expression ± SE of three independent experiments in triplicate is shown. The expression values of miR-290-295 cluster were converted into heat map using JColorGrid (15).

Gain and loss of function experiments. PRE SEN MEF were transfected with specified miRNAs or with miR-NC. Briefly, 15 µl OptiMEM and 25 µl transfection buffer plus 80 nM miRNA were mixed with a solution of Gene Silencer (5 µl) plus OptiMEM (25 µl). After 15 min incubation, OptiMEM was added up to 800 µl. Then, the transfection mixture was added to 1.5 × 10^5 cells seeded 24 h previously in 30 mm diameter dishes. After 6 h the medium was replaced with DMEM-HG +10% FBS. With this protocol >90% of PRE SEN MEF were transfected (data not shown). The d-100, d-125b, d-290, and d-NC were transfected using the same procedures. Cells were collected at 48 h posttransfection and used for the detection of SA-β-gal" cells and for the quantification of p19Arf, p16, and p21 expression. For the miR-290 depletion experiments during culture senescence (see Fig. 3E), a slightly modified transfection procedure was set up. Briefly, at the end of transfection (6 h) cells were collected and seeded at specific cell density for SA-β-gal" and p16 detection. With this protocol 70% of SEN MEF were transfected.

The protocol of combined treatment miR-290/NCZ, or d-290/NCZ was the following: 1.5 × 10^5 cells were seeded 24 h before transfection in 30 mm diameter dishes, and 6 h after transfection the medium was replaced with DMEM-HG + FBS. After another 6 h cells were trypsinized and 6 × 10^5 cells were seeded in 100 mm diameter dishes. Cells were further incubated for 24 h, and then NCZ was added. After 16 h exposure cells were collected and used for molecular and cellular assays as specified.

Real-time PCR analysis. Total RNA was extracted from 1 × 10^6 cells using the RNeasy mini kit. After DNase treatment, 1 µg of total RNA was retrotranscribed using SuperScript II reverse transcriptase following the manufacturer’s instruction. Real-time PCR (qRT-PCR) was carried out with LightCycler 480 (Roche). Taqman probes and oligonucleotides were used as follows: for p19Arf, forward (F) (5'-CATGGTGTCGAGGTCTTTG-3'), reverse (R) (5'-GCTGCTGTCCTGGTGCTTC-3') and probe (5'-CAGCTGAGGATTCTGGCGG-3'); for p16, F (5'-CGAAGGCGATAGCTCG-3') and probe (5'-CATGCTGGCAGATGCTC-3') and probe (5'-CAGAGGGCCACCTGGGTC-3'); for p21, F (5'-TCCACCGGATCTACGAC-3') and probe (5'-AGATGGTCGGAGCGCAC-3'); for RLF, F (5'-AATCGAGCCTGAAGAAACACTT-3') and probe (5'-CTGGGCGCATACAGGTCGAG-3'); for E2F1, F (5'-TGCACGAAGATCCTGAGT-3') and probe (5'-CTCAAGGCGCTTAC-3'); and probe (5'-CAGCCACA-3'). All reactions were performed in triplicate. Relative quantification of gene expression was calculated with the standard curve method. Transcript values were normalized with those obtained from the amplification of GAPDH (internal control) with the following primers: F (5'-GCTTCTCGGTCTCTCTACC-3') and R (5'-TGCTGCTCCACACCACTCT-3'), and probe (5'-CCTGGAGAAGACCTGGCAATG-3'). Each point represents the mean ± SE of at least three independent experiments.

Western blot analysis. Samples of 6 × 10^5 cells were lysed (20 mM Tris-HCl pH 8.0, 20 mM NaCl, 10% glycerol, 1% NP40, 10 mM EDTA, 2 mM PMSF, 2.5 µg/ml leupeptin). Proteins (30 µg/lane) were separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Immunoblots were probed with the following primary antibodies: anti-LRF (1:1,000), anti-E2F1 (1:1,000), anti-α tubulin (1:20,000). Signals were revealed after incubation with the recommended secondary antibody coupled to peroxidase using ECL. Scanned images were quantified using Scion Image software.

NCZ treatment. PRE SEN MEF were seeded at cell density of 6 × 10^5 per 100 mm diameter culture dish. After 48 h culture medium was replaced with medium containing NCZ (50 ng/ml), and 16 h later cells were collected and seeded to detect SA-β-gal" cells. Pellets of 1 × 10^6 cells were used for the determination of miRNAs and miRNAs levels.

Immunocytochemistry. Immunofluorescence microscopy was carried out on cells grown on coverslips. In brief, cells were fixed with paraformaldehyde 1% in PBS for 10 min and, after two PBS washes, incubated with a mix containing PH3 antibody (1:200), 1% BSA, and 0.1% Triton in PBS for 24 h at 4°C. Incubation with a mix containing the secondary antibody (1:500 of 488 Alexa Fluor anti-rabbit, 1% BSA, 0.1% Triton in PBS) was performed in the dark at room temperature for 2 h. Coverslips were washed twice for 10 min with PBS and covered with Hoechst (20 µg/ml, 1:2,000) for 15 min. Photographs were taken with a Leica DC 300F camera using an oil immersion Plan-Apochromat 63x/1.2 objective lens (Leica, DC 350 F). Images were processed with Adobe Photoshop 6.0 software. To determine PH3-positive nuclei, 25 consecutive fields (~500 cells) were screened.

Statistical analysis. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical differences were determined by unpaired t-test, with values of P < 0.05 considered statistically significant. Each experimental point in the graph represents the mean ± SE of at least three independent experiments. When necessary, the relative expression is reported.
RESULTS

Senescent MEF overexpress a specific subset of miRNAs. MEF were cultured according to the 6T3 protocol (see METHODS), and cell number as well as SA-β-gal+ cells were measured at each passage. The resulting curves show that cell yield decreases rapidly from passage 1 to passage 4 (Fig. 1A), thereafter remaining constant till passage 6; on the other hand, the percentage of SA-β-gal+ cells progressively increases (Fig. 1B). The transition of MEF from presenescence (passage 1) to senescence state (passage 6) was characterized at the molecular level. An important locus associated to culture senescence is INK4A, which codifies for two transcripts: p19ARF and p16INK4a (29). Both transcripts and in particular p16 increase with passage, while the culture becomes progressively senescent (Fig. 1C). Considering that the activation of the p53 pathway is a consequence of p19ARF upregulation, the expression of p21, a direct p53 target (16), was measured. The results show that the expression of p21 also increases with passage, paralleling the observed p19Arf increase (Fig. 1C). A DNA content analysis was also performed at each passage. The DNA content profiles show a progressive shift from 2C DNA content to 4C DNA content per cell from passage 1 up to passage 6 (Fig. 1D). This finding was not unexpected since an increased number of cells with a 4C DNA content, probably G1 tetraploid cells,
have been observed during culture as well as premature senescence induced by cellular stress (1, 8, 22). Based on these biological, biochemical, and molecular outcomes we define MEF as PRE SEN till passage 3 and as fully SEN at passage 6.

We have shown that an anti-senescence gene Lrf is under miRNA control (27); for this reason we wanted to verify whether the senescence process itself is regulated by miRNAs. To determine whether miRNA levels changes during senescence the global miRNA expression profile of PRE SEN was compared with that of SEN cells. The analysis of the miRNA microarray showed that eight miRNAs are expressed at higher levels (Z score >2 SD) and one is underexpressed (Z score <2 SD) in SEN cells (Fig. 1E).

**miR-290 is involved in culture senescence of MEF.** We previously reported that after transfection of MEF with either si-LRF, an siRNA specific for Lrf, or miR-20a, which inhibits the expression of Lrf, senescent-SA-β-gal+ cells are induced (27). Moreover, we showed that the transfection of either si-LRF or miR-20a, besides Lrf downregulation, induces the overexpression of different subsets of miRNAs (26).

To pin-point which of the senescence- and/or Lrf-associated miRNAs are directly involved in the senescence process, we searched for those miRNAs that are deregulated in all three situations. This approach revealed that miR-290 was overexpressed in all three subsets (Fig. 2A), so we focused our attention on this miRNA, aiming to unravel its role in MEF senescence. Firstly, we verified whether the time course of miR-290 expression correlates with culture senescence and found that it increases with passage to a greater extent than miR-100 and miR-125b, two miRNAs whose expression does not change after LRF downregulation (Fig. 2B) (26).

As miR-290 belongs to mmu-miR-290-295 cluster, we considered the cluster components (miR-291-3p, 292-3p, and 295) whose expression was under the cut-off (Z score >2 SD) of miRNA microarray. The analysis of qRT-PCR data showed increased miRNA expression during passage in culture, albeit less than miR-290 (Fig. 2C). MiR-292-5p, belonging to the cluster, was not quantified by qRT-PCR as we already showed its upregulation by microarray analysis (Fig. 1E).

This differential expression of miRNAs belonging to the same cluster is a known phenomenon (13, 24).

The observation that miR-290 expression increases when LRF decreases (by si-LRF and miR-20a) (Fig. 2A) prompted us to verify this correlation in the culture senescence model. Data clearly show that LRF expression diminishes along with passage, as does that of E2F1, a marker of cell proliferation (Fig. 2D). Therefore, LRF downregulation and miR-290 upregulation coincide with senescence and suggest a possible interconnection between these two events.

**Overexpression of miR-290 induces senescence, whereas miR-290 depletion inhibits senescence in PRE SEN MEF.**

As miR-290 is overexpressed in SEN MEF, we asked whether miR-290 is able to induce senescence. PRE SEN cells were transfected with miR-290 and the induction of SA-β-gal cells was measured. Compared with the unrelated miR-100 and miR-125b, miR-290 significantly enhances the frequency of SA-β-gal+ cells (Fig. 3A). To verify whether the induced senescence was a direct or indirect effect driven by miR-290, PRE SEN cells were transfected with d-290. The results showed that d-290 reduces the frequency of SA-β-gal+ cells compared with control (Fig. 3B) and stimulates cell proliferation (Fig. 3C). Then we checked whether the miR-290-induced senescence correlates with the transcriptional activation of the Ink4A locus and the consequent increase of the two encoded proteins p19ARF and p16INK4a (29). MiR-290-transfected cells compared with miRNA control (27); for this reason we wanted to verify this correlation in the culture senescence model. Data clearly show that LRF expression diminishes along with passage, as does that of E2F1, a marker of cell proliferation (Fig. 2D). Therefore, LRF downregulation and miR-290 upregulation coincide with senescence and suggest a possible interconnection between these two events.

As p19ARF protein upregulates p53 level by inhibiting MDM2 (25) we determined the transcription of p21, which is the downstream target of p53 (Fig. 3D) (16). We found that p21 is similarly expressed in MEF transfected with miR-290 and in control cells, suggesting that the axis p19ARF-p53-p21 is not fully activated.

To be more confident on the antisenescence role of d-290, MEF before (passages 1 and 3) and after (passages 5 and 7) the blockage of cell proliferation (Fig. 3E) were transfected with either d-NC or d-290. At passage 1 d-290 is unable to modify the frequency of SA-β-gal+ cells, probably because miR-290 is expressed at very low level so that its depletion has no effect. At passage 3 d-290 reduces the frequency of SA-β-gal+ cells, whereas its effect diminishes at passages 5 and 7 (Fig. 3F).
We then quantified the p16 transcript and found that p16 decrease correlates with the efficiency of d-290 in reducing the SA-/H9252/H11001 cells (Fig. 3F). To exclude the possibility that the failed reduction of both SA-/H9252/H11001 and p16 expression observed at passages 5 and 7 was due to a differential susceptibility to transfection, we transfected an oligo-FITC, and thereafter we measured the frequency of fluorescent cells. We observed that 95% (MEF at passages 1 and 3) and 70% (MEF at passages 5 and 7) were positive to fluorescence, respectively (Fig. 3E), suggesting that the transfection efficiency may not play an important role.

The senescence induced by NCZ resembles culture senescence. We found that the culture senescent phenotype of MEF is also characterized by an increasing amount of arrested cells with a 4C DNA content, likely tetraploid G1 cells (8). A growing body of evidence indicates that whenever polymerization of spindle microtubules is affected, the spindle and mitotic check points are activated (17, 22) so that primary cells become irreversibly arrested as G1 tetraploid cells prone to senescence (21). We then assayed the ability of NCZ, a drug that inhibits spindle microtubules polymerization and permanently arrests primary fibroblasts in tetraploid G1 (1, 2), to induce senescence. PRE SEN MEF were exposed to NCZ and screened for the DNA content profile and for the amount of SA-/H9252/H11001 cells. The results show that cells with 4C DNA content are twice as frequent (Fig. 4C) and SA-/H9252/H11001 cells are induced following NCZ treatment (Fig. 4D). To verify whether cells with a 4C DNA content were tetraploid G1 rather than G2/M, the expression of PH3, which marks mitotic cells (31), was evaluated. The results show that the percentage of PH3-positive cells is considerably lower in NCZ-treated cells than that found in untreated cells, strongly indicating that the majority of NCZ-treated cells are in G1 rather than in G2/M (Fig. 4, A and B). These findings suggested that NCZ induced senescence by mechanisms resembling culture senescence where proliferating cells exit mitosis, become arrested as tetraploid G1 cells, and undergo senescence. We then determined the time course of miR-290 accumulation together with the other culture senescence markers (p16, Lrf, and E2fl) (Fig. 4E). The resulting curves showed that miR-290 and p16 are stably upregulated, while Lrf and E2fl are transiently downregulated following NCZ exposure. Thus we decided to use the
NCZ treatment-induced senescence as an additional model to study the role of miR-290 in senescence.

Depletion of miR-290 abolishes the senescence induced by NCZ. To investigate whether miR-290 was causative of NCZ-induced senescence, PRE SEN cells were transfected with either miR-290 or d-290 and soon after exposed to NCZ (Fig. 5A). Our results show that miR-290 alone but not miR-NC is able to induce the same amount of SA-β-gal'' cells as NCZ. However, neither miR-290 nor miR-NC enhances the NCZ effect; vice versa, d-290 significantly inhibits NCZ-induced SA-β-gal'' cells. Notably, the depletion of miR-290 by d-290 did not abolish the G2/M accumulation due to NCZ (Fig. 5B) while it abolished the overexpression of p16 induced by NCZ treatment (Fig. 5C). These findings suggest that while the cell cycle block imposed by microtubule disruption is miR-290 independent, senescence and p16 increase, which accompanies the cell cycle block, is miR-290 dependent.

DISCUSSION

A cell is usually considered senescent when it stops proliferating even though it remains metabolically active, acquires a flat morphology, and becomes SA-β-gal'' (12). We used the culture senescence of MEF as a model to investigate whether the transition of MEF from presenescence to senescence is accompanied by changes in the expression of specific miRNAs and whether these miRNAs have a role in inducing senescence. The analysis of the molecular and cellular changes during the transition of MEF from presenescence to senescence revealed that the senescent phenotype was characterized predominantly by a 4C DNA content per cell, overexpression of the SA gene set (p19Arf, p16, p21), and increase of SA-β-gal'' cells (Fig. 1, A–D).

MiR-290 was identified as a miRNA with a potentially important role in senescence as it was upregulated in senescent cells obtained by three different methods (culture induced, miR-20a, or si-LRF-induced senescence). During culture-induced senescence the time course of miR-290 expression correlates well with that of SA genes (p19Arf, p16, p21), and increase of SA-β-gal'' cells (Fig. 1, A–D).

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also by chromatin modification. The importance of chromatin modifications is also inferred by the role of LRF in the induction of MEF senescence. LRF transcriptionally represses p19Arf by recruiting histone deacetylases (20), and in this paper we show for the first time that this repressor steadily decreases with passage, so it may be considered an additional marker of MEF senescence (Fig. 2D). Moreover, as the expression of miR-290 negatively correlates with that of this transcriptional repressor a direct or indirect control of LRF on this miRNA can be hypothesized. MiR-290 gain and loss of function experiments clearly demonstrate that this miRNA is causatively related to senescence. However, this is true only during the presenescence state; the experiments with d-290 indicate that proliferating cells are available (Fig. 3F). We know that MEF at passages 5 and 7 are mostly nonproliferating cells. The finding that miR-290 depletion is less effective at passages 5 and 7 strongly supports the hypothesis that miR-290 suppression protects only proliferating cells from senescence, while cells already committed to the senescence pathway cannot revert to a proliferative state.

To get more insights into the role of miR-290 we focused on NCZ-induced senescence, which in many aspects resembles culture senescence. An interesting outcome of our study is that NCZ treatment of PRE SEN MEF, besides inducing ploidy changes (Fig. 4C) and SA-β-gal+ cells (Fig. 4D), increases miR-290 and p16 expression (Fig. 4E), as found in culture senescence (Figs. 2B and 1C). Remarkably, the combined d-290/NCZ treatment abolished the induction of senescence (Fig. 5A) and the overexpression of p16 (Fig. 5C) but not the G2/M block (Fig. 5B). These results indicate that miR-290 acts as a physiological effector of senescence induced by NCZ, and it may be necessary to perceive the cell cycle block as a senescence signal. Intriguingly, the cell cycle block induced by NCZ appears to be miR-290 independent. As in culture senescence, NCZ induces a transient downregulation of Lrf and E2f1 and a stable upregulation of miR-290 and p16, further reinforcing the hypothesis of a possible connection between Lrf and miR-290.

How does miR-290 induce senescence? A recurrent result found in culture-, miR-290-, or NCZ-induced senescence is the consistent association between miR-290 and the increased expression of the Ink4A locus (p16 and p19Arf). It is an emerging notion that p16 pathway is primarily responsible for premature senescence induced by stress while the p53-p21-pRb pathway mediates senescence induced by telomere shortening (38). The upregulation of p16 is in keeping with our previous findings showing that it is also at the basis of the miR-20a-induced senescence via LRF downregulation (27). The increase of p16 induced by transfecting miR-290 (Fig. 3D) is much smaller than that observed during culture senescence.
At present, we do not know whether or how miR-290 upregulates p16 and to a lesser extent, p19ARF, but in silico analysis indicates enhancer of zeste (EZH2), a component of the polycomb repressive complex 2 (PRC2), as a possible target of miR-290. As EZH2 has been shown to repress Ink4A transcription and to decrease during MEF senescence (9) it might be an ideal candidate target of miR-290. It is becoming clear that miRNAs affect multiple targets; indeed, other interesting predicted targets of miR-290 are different members of the MAPK family such as MAPK1/ERK1, known to play an important role in cell proliferation (23). Thus, it is possible that miR-290 drives cells toward senescence by the combined downregulation of these protein kinases, together with the upregulation of inhibitors of the cell cycle (p19ARF and p16).

In summary, our data indicate that miR-290 in MEF is causatively implicated in both culture-induced as well as NCZ-induced senescence, thus expanding the physiological role of this important miRNA previously reported as an embryonic stem cell-specific miRNA. It is interesting to note that miR-290 appears to be upregulated in connection with LRF downregulation, raising the interesting possibility that LRF, directly or indirectly, controls this miRNA. Moreover, miR-290 upregulation is always accompanied by the upregulation of the Ink4A locus (especially p16). In this regard it will be interesting to establish whether there is a direct link between miR-290 and the Ink4A locus via inhibition of repressors/...


